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C5L2 Regulates DMP1 Expression during Odontoblastic Differentiation

F. Chmilewsky¹, R. Liang¹, M. Kanazawa¹, I. About², L.F. Cooper¹, and A. George¹

Abstract

The presence of stem cells within the dental-pulp tissue as well as their differentiation into a new generation of functional odontoblast-like cells constitutes an important step of the dentin-pulp regeneration. Recent investigations demonstrated that the complement system activation participates in 2 critical steps of dentin-pulp regeneration: pulp progenitor's recruitment and pulp nerve sprouting. Surprisingly, its implication in odontoblastic differentiation has not been addressed yet. Since the complement receptor C5a receptor-like 2 (C5L2) is expressed by different stem cells, the aim of this study is to investigate if the dental pulp stem cells express C5L2 and if this receptor participates in odontoblastic differentiation. Immunohistochemistry performed on human third molar pulp sections showed a perivascular co-localization of the mesenchymal stem cell markers STRO1 and C5L2. In vitro immunofluorescent staining confirmed that hDPSCs express C5L2. Furthermore, we determined by real-time polymerase chain reaction that the expression of C5L2 is highly modulated in human dental pulp stem cells (hDPSCs) undergoing odontoblastic differentiation. Moreover, we showed that this odontogenesis-regulated expression of C5L2 is specifically potentiated by the proinflammatory cytokine TNF α . Using a C5L2-siRNA silencing strategy, we provide direct evidence that C5L2 constitutes a negative regulator of the dentinogenic marker DMP1 (dentin matrix protein 1) expression by hDPSCs. Our findings suggest a direct correlation between the odontoblastic differentiation and the level of C5L2 expression in hDPSCs and identify C5L2 as a negative regulator of DMP1 expression by hDPSCs during the odontoblastic differentiation and inflammation processes. This work is the first to demonstrate the involvement of C5L2 in the biological function of stem cells, provides an important knowledge in understanding odontoblastic differentiation of dental pulp stem cells, and may be useful in future dentin-pulp engineering strategies.

Keywords: stem cell, reparative dentinogenesis, dentin matrix protein 1, pulp biology, inflammation, TNF-alpha

Introduction

It is established that the dentin-pulp complex has a high regeneration capacity. This varies from a "simple" upregulation of the underlying odontoblast synthetic activity, which leads to regenerating a protective reactionary dentin, to more severe dentin-pulp injuries. These usually imply complete dentin-pulp regeneration and seem to be directly correlated with the presence of stem cells within the dental pulp (About et al. 2000; Gronthos et al. 2002). Carious lesions or deep cavity preparations may lead to odontoblast destruction (Smith et al. 1995; Goldberg et al. 2008). However, a new generation of odontoblast-like cells is responsible for reparative dentin apposition within the pulp chamber (Fitzgerald et al. 1990; Shi and Gronthos 2003). This process, which depends on the differentiation of the dental pulp stem cells (DPSCs) into odontoblast-like cells, is subsequent to an inflammatory reaction (Mitsiadis et al. 2008).

The complement system (CS) is an important component of innate immunity that plays essential roles in inflammation and immune defense mechanisms (Ricklin et al. 2010). Its activation as a proteolytic cascade is one of the most effective initial mechanisms for eliminating invading pathogens and altered

host cells (Walport 2001). Recent investigations demonstrated that CS is activated in the dentin-pulp complex of human carious teeth (Chmilewsky et al. 2013; Chmilewsky et al. 2014) and participates in the elimination of cariogenic bacteria (Jeanneau et al. 2015).

It has long been established that inflammation is required for efficient tissue regeneration (Kloner et al. 1978). Accordingly, several studies demonstrated that CS is essential for tissue regeneration (Ignatius et al. 2011; Lara-Astiaso et al. 2012). In a mouse model of liver regeneration following CCL4-induced toxic damage (Mastellos et al. 2001), mice deficient in the fifth component of complement C5 exhibited impaired liver regeneration. The regeneration process was

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A supplemental appendix to this article is available online.

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restored by a C5 injection. Accordingly, several recent investigations identified the CS as a source of regeneration signals for dentin-pulp regeneration by participating in 2 critical steps: the pulp progenitor's and fibroblast's recruitment to the injured site and the pulp nerve sprouting beneath carious injury (Chmilewsky et al. 2013; Chmilewsky et al. 2016; Rufas et al. 2016). However, to date, there exist no investigations implicating the CS in DPSC odontoblastic differentiation (OD) and/or tertiary dentin formation.

The reported role of the CS in tissue regeneration seems mainly mediated by the active fragment C5a and its receptor, C5aR (Monk et al. 2007; Laursen et al. 2012). Accordingly, the interaction between C5aR and C5a has been identified as an important event of the dentin-pulp regeneration under carious injury, by inducing the recruitment of DPSCs and the neurite outgrowth to the injured site (Chmilewsky et al. 2013). Interestingly, a second receptor for C5a, the C5a receptor-like 2 (C5L2), has received much less attention, and its biological functions remain enigmatic (Ohno et al. 2000; Li et al. 2013). Unlike most G protein-coupled receptors, C5L2 is deficient in G-protein coupling. Yet, C5L2 is emerging as a functional receptor with important roles in inflammation (Bamberg et al. 2010). For example, the expression of C5L2 is increased in the inflamed area of human carious tooth sections, implying a role for C5L2 in the direct pulpal response to caries (Chmilewsky et al. 2017). Although C5aR is known to be expressed in similar tissue and cellular locations as C5L2 and is expressed by hDPSCs (Li et al. 2013), C5L2 expression by DPSCs has not been investigated. Here, we set to investigate if DPSCs express the C5L2 and to check whether this receptor is involved in OD.

Materials and Methods

Primary Cell Cultures

Human pulp cells were prepared from immature third molars at the two-thirds root formation stage by the explant outgrowth method and routinely cultured as described previously (About et al. 2000; Chmilewsky et al. 2014).

Human DPSCs (hDPSCs) were directly sorted from pulp cell cultures at passages 1 to 3 with a mouse anti-human STRO1 antibody coupled with immune magnetic beads according to the manufacturer's protocol (Dyna) and characterized as described previously (Chmilewsky et al. 2014; Chmilewsky et al. 2015). Thus, only STRO1-positive cells also positive for CD44, CD90, CD105, CD146, and CD146 are designated thereafter as hDPSCs.

Human bone marrow-derived mesenchymal stem cells (hBMMSCs) were purchased from Lonza and routinely cultured in the recommended growth medium.

Tissue Collections

Human immature third molars, freshly extracted for orthodontic reasons, were obtained in compliance with French legislation (informed patients' consent and institutional review board

approval of the protocol used). Teeth were fixed and processed as described previously (Téclès et al. 2005).

Tissue sections were generated from 5-d-old wild-type and dentin matrix protein 1 (DMP1)-overexpressing mouse heads. All mice were generated and maintained according to the Animal Care Committee of the University of Illinois at Chicago. All studies were performed per the university's protocol (animal assurance 13-210).

Immunohistochemistry

See Appendix 1.

Immunofluorescence Staining

See Appendix 1.

Real-time Polymerase Chain Reaction

See Appendix 1.

C5L2 Silencing by siRNA

Transient transfection of hDPSCs with appropriate siRNA (as indicated) was performed with the siRNA Reagent System (sc-45064) according to the manufacturer's protocol (Appendix 1). The efficiency of C5L2 silencing is shown in Appendix Figure.

Statistical Analysis

All experiments were performed in triplicate. The data were calculated as mean \pm SD. Statistical analyses were performed with Student's *t* test or analysis of variance. Statistical significance was defined as $P \leq 0.05$.

Results

Mesenchymal Stem Cell Marker STRO1 and C5L2 Were Co-localized in Human Dental Pulp

In the pulp of human teeth, the cells positive for STRO1 were observed in the perivascular area, and C5L2 staining was detected in all the pulp area (Fig. 1A). On merged images, STRO1 and C5L2 were co-localized in the same perivascular area (Fig. 1A, arrowhead), and higher magnifications of the merged images indicated that STRO1 and C5L2 are coexpressed by the same cells (magnified boxed area). These data suggest that hDPSCs express the complement receptor C5L2.

Cultured hDPSCs Expressed C5L2

Immunofluorescence double staining was performed on cultured hDPSCs. Our data confirmed STRO1 expression in hDPSCs (Fig. 1B) and revealed that they all express C5L2 (Fig. 1B, middle panel) as confirmed on STRO1 and C5L2 merged

images (Fig. 1B, merge). The coexpression of STRO1 and C5L2 is clearly visible at higher magnification (Fig. 1B, magnification of the boxed area).

C5L2 Expression Was Modulated during hDPSC OD

The analysis of the C5L2 expression by real-time polymerase chain reaction (PCR; Fig. 2A) indicated that, in contrast to stable C5L2 levels in undifferentiated hDPSCs, C5L2 expression is elevated in hDPSCs cultured in dentinogenic medium (DM). Specifically, after only 4 h in DM, C5L2 mRNA levels significantly increased and remained elevated up to 3 d. The most significant increase in C5L2 expression was detected between 7 and 14 d in DM. After 21 d in DM, the C5L2 mRNA level detected was drastically reduced. Interestingly, very little changed in the expression of the second C5a receptor. C5aR was observed during hDPSC OD (Fig. 2B). Therefore, the modulations of C5L2 expression in hDPSCs undergoing differentiation suggests a role for this receptor in this process. Moreover, the odontogenesis-regulated expression of C5L2 versus C5aR suggests an independent function of C5L2 in odontogenesis.

Effect of Inflammation on hDPSC C5L2 Expression

We evaluated the effect of 5 inflammatory components, known to be implicated in the carious process, in the expression of C5a receptors by hDPSCs (Fig. 2B). As reported earlier, C5L2 expression increased in hDPSCs undergoing OD. While LPS, LTA, IL6, and C5a did not affect the level of C5L2 mRNA in hDPSCs, TNF α specifically increased C5L2 expression in hDPSCs undergoing OD. Interestingly, none of these inflammatory components affected C5aR expression in hDPSCs (Fig. 2B). These data indicated that the expression of C5L2 in hDPSCs undergoing OD was potentiated by TNF α and supported the C5aR-independent function of C5L2 in odontogenesis.

TNF α Increased the Expression of C5L2 during hDPSC OD

Further real-time PCR analysis revealed that the odontogenesis-regulated expression of C5L2 was quickly potentiated by TNF α stimulation since a significant increase of C5L2

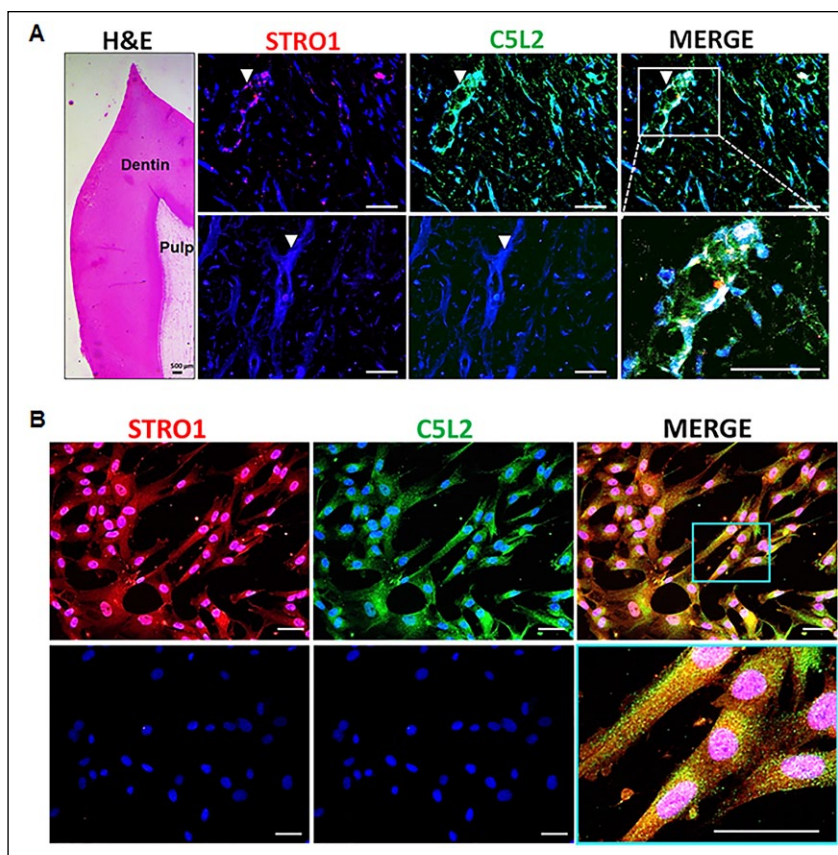


Figure 1. Expression of C5L2 and STRO1 in vivo. **(A)** Immunofluorescence double staining was used to localize STRO1- and C5L2-expressing cells within the same human tooth section. While the expression of STRO1 was detected in the vicinity of blood vessels, C5L2 is detected in all pulp tissue. Merged images of STRO1 and C5L2 staining demonstrated a coexpression of C5L2 and STRO1 in the perivascular area, which is clearly visible at a higher magnification. Negative controls of STRO1 and C5L2 performed by replacement of the primary antibodies with isotype controls showed no labeling. Scale bars: 50 μ m. Nuclei were counterstained with DAPI (blue). H&E, hematoxylin-eosin. Arrowheads indicate blood vessels. Boxed area is magnified and shown below. **(B)** Expression of C5L2 by hDPSCs in vitro. Immunofluorescence double staining was used to analyze C5L2 expression in hDPSCs. All hDPSCs express both the mesenchymal stem cell marker STRO1 and the complement receptor C5L2. Merged images of STRO1 and C5L2 revealed that all STRO1-positive cells expressed C5L2 and are clearly visible at higher magnification of the boxed area in the upper panel. No STRO1 and C5L2 immunostaining was observed with the negative controls performed by replacement of the primary antibodies with isotype controls on hDPSCs. Scale bars = 50 μ m. Nuclei were counterstained with DAPI (blue). hDPSC, human dental pulp stem cell.

expression was detected after only 6 h of TNF α treatment in DM (Fig. 2C). Interestingly, we observed that the TNF α -potentiated C5L2 expression was sustained with increasing concentration of TNF α treatment, as observed at 24 h of culture (Fig. 2C top panel). In addition, while an increase of C5L2 expression in hBMMSCs undergoing osteogenic differentiation was detected at the 24-h culture time (Fig. 2C), TNF α treatments of hBMMSCs did not affect the level of C5L2 expression during the differentiation process, regardless of the concentration used (Fig. 2C bottom panel). Taken together, these results indicated that C5L2 expression levels in response to TNF α depended on the organ/tissue considered and so pinpointed the importance of both the “inflammation parameter” and the targeted cell type in dentin-pulp regeneration.

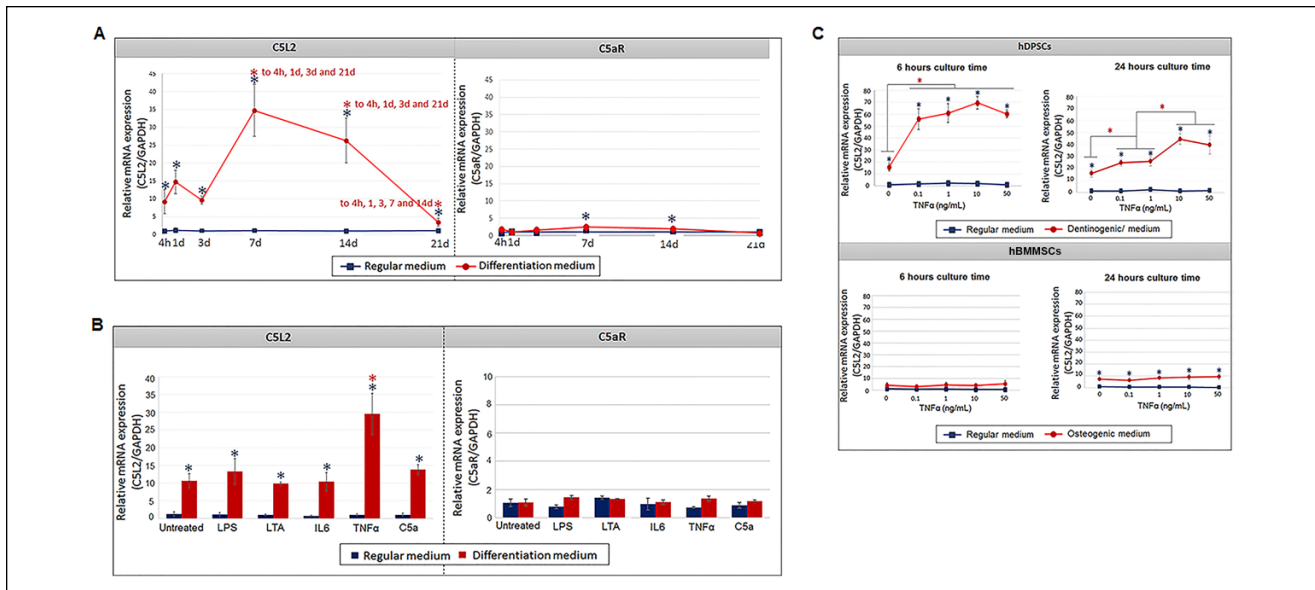


Figure 2. Time-course analyses of C5L2 and C5aR mRNA expression in hDPSCs. **(A)** Real-time polymerase chain reaction (PCR) was used to evaluate the level of C5L2 and C5aR mRNA in hDPSCs after 4 h and 1, 3, 7, 14, and 21 d of culture in regular medium (blue lines) or dentinogenic medium (red lines). In contrast to stable C5L2 levels in undifferentiated hDPSCs, C5L2 expression quickly increased in dentinogenic medium, with the most significant increase detected between 7 and 14 d (fold increase: 7 d = 34.7 ± 7.3 , 14 d = 26.3 ± 6.3). Comparatively, C5aR expression changed little during odontoblastic differentiation of hDPSCs (blue vs. red line). Results are expressed as relative expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as mean \pm SEM of 3 independent experiments. $^*P < 0.05$ vs. regular medium at the same time point, $^{\#}P < 0.05$ vs. dentinogenic medium at indicated time points. **(B)** Effect of inflammatory components on the expression of C5a receptors. Real-time PCR was used to determine the effect of 5 inflammatory components (LPS, LTA, IL6, TNF α , and C5a) on the level of C5L2 and C5aR mRNA in hDPSCs after 1 d of culture in regular medium (blue bars) or dentinogenic medium (red bars). While these inflammatory components do not affect the expression of C5aR in regular or dentinogenic medium, TNF α stimulation specifically increases C5L2 expression in hDPSCs undergoing odontoblastic differentiation (red bar "TNF α " vs. red bar "Untreated"). Results are expressed as relative expression to GAPDH. Data are presented as mean \pm SEM of 3 independent experiments. $^*P < 0.05$ vs. regular medium, $^{\#}P < 0.05$ vs. dentinogenic medium and without inflammatory stimulation. **(C)** Effect of TNF α on C5L2 expression. hDPSCs or hBMMSCs were cultured in regular or dentinogenic/osteogenic medium and with 0, 0.1, 1, 10, or 50 ng/mL of TNF α . C5L2 mRNA expression was determined by real-time PCR analysis after 6 and 24 h of culture. TNF α stimulation, independent of the concentration used, quickly potentiates the odontogenesis-regulated expression of C5L2. However, this C5L2 expression increase is sustained with increasing concentration of TNF α treatment even at 24 h. An increase of C5L2 expression in hBMMSCs undergoing osteogenic differentiation is detected at 24 h but not at 6 h of culture time. The treatments of hBMMSCs with TNF α do not affect the level of C5L2 expression during the differentiation process, regardless of the concentration used. Results are expressed as relative expression to GAPDH. Data are presented as mean \pm SEM of 3 independent experiments. $^*P < 0.05$ vs. regular medium at the same TNF α concentration, $^{\#}P < 0.05$ vs. dentinogenic medium. hBMMSC, human bone marrow-derived mesenchymal stem cell; hDPSC, human dental pulp stem cell.

Expression of C5L2 Was Detected in Mouse Teeth and Correlated with DMP1 Expression

Immunofluorescence double staining performed on 5-d mouse head sections confirmed DMP1 overexpression in the teeth of DMP1 transgenic mice (Fig. 3). Indeed, DMP1 labeling was more intense in the dental pulp of genetically modified mice than in that of the wild-type mice. Moreover, our data clearly indicated that C5L2 was expressed in the pulp cells of mouse teeth. This is the first report demonstrating the expression of C5L2 in mouse dental pulp. Interestingly, the C5L2 staining appeared weaker in the dental pulp of wild-type mice than in the pulp of mice overexpressing DMP1 (panel marked C5L2), which showed an intense red staining. Merged figures of DMP1 and C5L2 staining demonstrated that DMP1 and C5L2 were expressed by the same cells in the dental pulp. These data could suggest a possible link between DMP1 expression and C5L2.

C5L2 Negatively Regulated the Expression of DMP1 in hDPSCs

The quantification of DMP1 was observed by real-time PCR on hDPSCs transfected with control or C5L2 siRNA after 24-h incubation in regular medium or DM (Fig. 4A). The expression of DMP1 increased after 24 h in DM (Fig. 4B). Importantly, the silencing of C5L2 significantly increased the expression of DMP1 in hDPSCs cultured in DM (Fig. 4A). In the presence of TNF α , which previously demonstrated elevated C5L2 expression (Fig. 2C), the silencing of C5L2 potentiated the increase of DMP1 expression by hDPSCs cultured in DM (Fig. 4A). These data indicated that C5L2 constituted a negative regulator of the DMP1 expression by hDPSCs undergoing OD. Moreover, this negative regulation appeared significantly higher in an inflammatory environment.

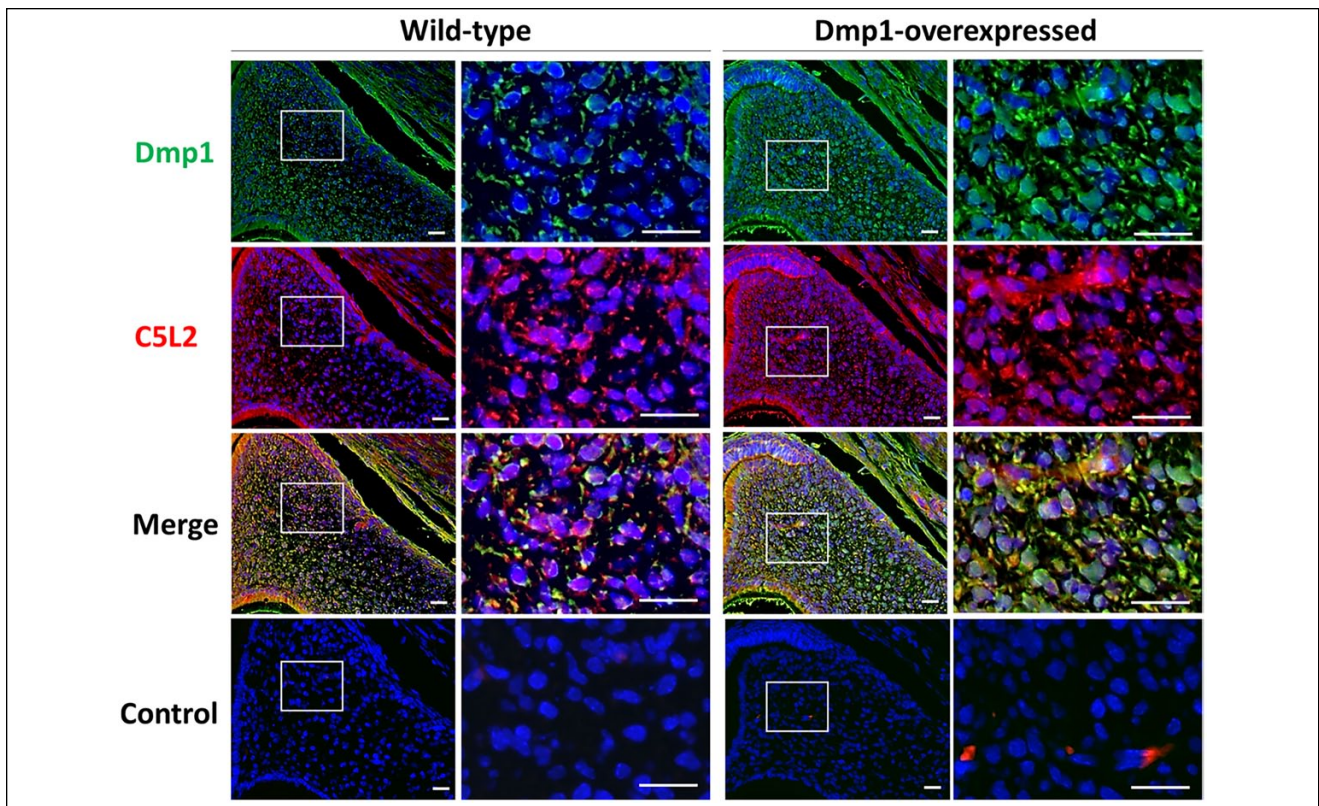


Figure 3. Detection of C5L2 and DMP1 expression in mouse tooth sections. Immunofluorescent double staining was used to investigate the expression of C5L2 in the dental pulp of wild-type mice and mice genetically modified for overexpression of DMP1. Our staining confirmed the DMP1 overexpression in the genetically modified mice, as revealed by the weaker DMP1 labeling in wild-type mice when compared with the DMP1-overexpressing mice sections. Similarly, in wild-type mice, the C5L2 detected in dental pulp is weaker than that observed in genetically modified mice. The merged image of DMP1 and C5L2 staining clearly demonstrated that DMP1 and C5L2 are expressed by the same pulp cells. Negative controls, performed by replacement of the primary antibodies with isotype controls in wild-type mouse sections and in DMP1-overexpressed mouse sections, showed no labeling (control). Boxed areas are magnified in the corresponding adjacent sections. Nuclei were counterstained with DAPI (blue). Scale bars = 50 μ m.

DMP1 Upregulated the Expression of C5L2 in hDPSCs

Interestingly, our real-time PCR data demonstrated that the level of C5L2 expression in hDPSCs was significantly increased after 24 h of DMP1 stimulation (Fig. 4B). These data indicated that DMP1 constituted a positive regulator of the C5L2 expression by hDPSCs, suggesting a regulatory loop between C5L2 and DMP1.

TNF α Increased the Expression of DMP1 in Undifferentiated hDPSCs

Further real-time PCR analysis revealed that TNF α significantly increased the expression of DMP1 in undifferentiated hDPSCs (Fig. 5A). Interestingly, TNF α had no effect on the level of DMP1 expression in hDPSCs undergoing OD, with a level of DMP1 comparable to the one detected in undifferentiated hDPSCs in the absence of TNF α stimulation (Fig. 5A).

C5a Stimulation Did Not Affect the Expression of DMP1 in hDPSCs

In contrast to the preceding observations regarding C5L2, the stimulation of hDPSCs with recombinant human C5a had no effect on the expression of DMP1 (Fig. 5B) in regular medium or DM. These results suggest that the modulation of the DMP1 expression in hDPSCs was independent of the C5a signaling.

Discussion

In this study, we identified the complement receptor C5L2 as a potential critical modulator of the hDPSC OD. Our *in vivo* data revealed that C5L2 is co-localized with the STRO1-positive cells in the pulp of human teeth. *In vitro*, our immunofluorescent double staining confirmed that hDPSCs express C5L2. Furthermore, we determined by real-time PCR that the expression of C5L2 is highly modulated in hDPSCs during the OD and that this C5L2 expression is specifically increased by the

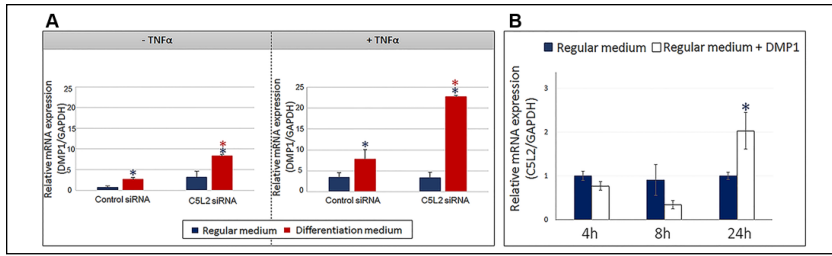


Figure 4. Effect of C5L2 silencing on the expression of DMP1 by hDPSCs. **(A)** Real-time polymerase chain reaction was used to determine the level of DMP1 mRNA in hDPSCs transfected with control or C5L2 siRNA and cultured 24 h in regular or dentinogenic medium \pm 10 ng/mL of TNF α . The DMP1 expression increased after 24 h in dentinogenic medium (red vs. blue bars of “Control siRNA”). The silencing of C5L2 significantly increased the expression of DMP1 (red bars of “C5L2 siRNA” vs. all the other bars). In the presence of TNF α , the silencing of C5L2 potentiated the increase of DMP1 expression by hDPSCs cultured in dentinogenic medium (red bars of “C5L2 siRNA”). Results are expressed as relative expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as mean \pm SEM of 3 independent experiments. * P < 0.05 vs. regular medium of the same transfection condition, * P < 0.05 vs. dentinogenic medium of the hDPSCs transfected with control siRNA. **(B)** Effect of DMP1 stimulation on C5L2 mRNA expression in hDPSCs. hDPSCs were cultured in regular medium with or without recombinant human DMP1 (250 ng/mL). C5L2 mRNA expression was determined by real-time polymerase chain reaction analysis after 4, 8, or 24 h of culture time. Results are expressed as relative expression to GAPDH. Data are presented as mean \pm SEM of 3 independent experiments. * P < 0.05 vs. regular medium. hDPSC, human dental pulp stem cell.

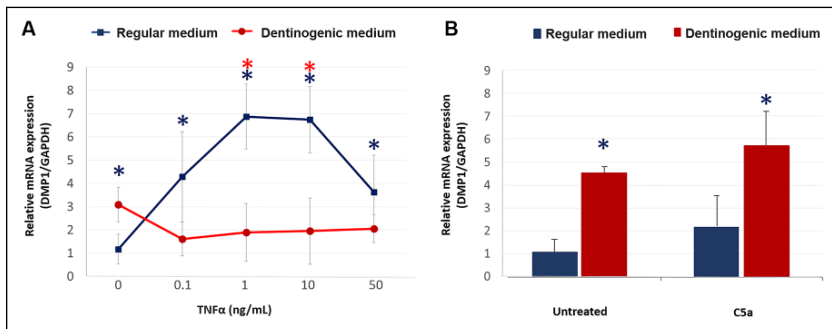


Figure 5. Effect of TNF α on DMP1 expression. **(A)** hDPSCs were cultured in regular or dentinogenic medium with 0, 0.1, 1, 10, or 50 ng/mL of TNF α . DMP1 mRNA expression was determined by real-time polymerase chain reaction analysis after 24 h of culture time. While TNF α stimulation significantly increases the DMP1 expression in hDPSCs cultured in regular medium, it abolished the DMP1 upregulation in hDPSCs cultured in dentinogenic medium. Results are expressed as relative expression to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as mean \pm SEM of 3 independent experiments. * P < 0.05 vs. regular medium at 0 ng/mL of TNF α , * P < 0.05 vs. dentinogenic medium. **(B)** Effect of C5a stimulation on DMP1 mRNA expression in hDPSCs. hDPSCs were cultured in regular or dentinogenic medium and with or without recombinant human C5a (1 nM). DMP1 mRNA expression was determined by real-time polymerase chain reaction analysis after 24 h of culture time. Results are expressed as relative expression to GAPDH. Data are presented as mean \pm SEM of 3 independent experiments. * P < 0.05 vs. regular medium. hDPSC, human dental pulp stem cell.

proinflammatory cytokine TNF α . Finally, using a C5L2-siRNA silencing strategy, we provide direct evidence that C5L2 constitutes a negative regulator of the DMP1 expression by the hDPSCs undergoing OD.

The dentin-pulp regeneration is a complex process requiring the presence of stem cells as well as their differentiation into a new generation of odontoblast (Fitzgerald et al. 1990; Gronthos et al. 2002; Iohara et al. 2008). It has long been established that dental pulp contains stem cells capable of dentin

regeneration (About et al. 2000). Although other localizations and niches cannot be excluded, several studies reported that the DPSCs reside in the perivascular area (Shi and Gronthos 2003; Têclès et al. 2005; Iohara et al. 2008). In line with these data, our immunohistochemistry confirmed the expression of the mesenchymal stem cell marker STRO1 by cells localized in the perivascular area of human dental pulp. Recent investigations demonstrated that the STRO1-positive cells present in the dental pulp tissues are sensitive to the CS activation occurring under carious injuries, by migrating according to a C5a gradient (Chmilewsky et al. 2013; Chmilewsky et al. 2014). The recruitment of these stem cells on the injured site, which constitutes one of the early and essential steps of dentin-pulp regeneration, occurs through the interaction between the complement active fragment C5a and its receptor C5aR. Interestingly, the second receptor for C5a, C5L2, is known to be expressed in similar tissue and cellular locations as C5aR; however, its expression by DPSCs has not been investigated (Li et al. 2013). Our immunofluorescent staining demonstrated that the C5L2 is localized in all the pulp tissue of human teeth. The expression of C5L2 in pulp fibroblasts is in line with previous reports showing that C5L2 represses brain-derived neurotrophic factor secretion in lipoteichoic acid-stimulated pulp fibroblasts (Chmilewsky et al. 2017). However, its expression in STRO1-positive cells is an original finding. This is demonstrated on tooth sections where STRO1-positive cells in the perivascular area also express the C5L2 and confirmed in vitro by immunofluorescence double staining and real-time PCR. Although another study reported that human embryonic stem cells and human-induced pluripotent stem cells express C5L2, its implication in the biological functions of stem cells has not been addressed yet (Hawksworth et al. 2014).

C5a is known as a powerful chemotactic factor for the cells that express the C5aR (Klos et al. 2009). Mainly known to recruit the immune cells, C5a also constitutes a chemotactic factor for stem cells (Schraufstatter et al. 2009; Chmilewsky et al. 2013). However, it was demonstrated that the interaction between C5a and its second receptor C5L2 does not stimulate chemotaxis (Okinaga et al. 2003). This raises the question of the C5L2 biological function in stem cells. Surprisingly, our real-time PCR results indicated that the expression of C5L2 is highly modulated in the hDPSCs

undergoing OD, suggesting an implication of this receptor in the OD process. C5L2—which is the second high-affinity receptor for C5a, identified after C5aR—is a 37kDa protein belonging to the superfamily of G protein-coupled receptor (Ohno et al. 2000; Li et al. 2013). Unlike most G protein-coupled receptors, C5L2 is deficient in heterotrimeric G protein coupling. Therefore, although C5L2 is weakly phosphorylated following its interaction with the anaphylatoxin C5a, no significant activation of MAP kinases and calcium flux was detected (Okinaga et al. 2003). Thus, C5L2 is often considered a “simple” modulator of the signaling and biological functions induced by C5aR-C5a interaction (Lee et al. 2008). The present study, showing an odontogenesis-regulated expression of C5L2 versus C5aR, could suggest a C5aR-independent function of C5L2 in hDPSC differentiation.

Like other stem cells, hDPSCs are resting cells residing beside differentiated cells within an intact and functional tissue, and they acquire their regenerative function only after tissue injury or pathologic conditions (About 2013). In dental-pulp regeneration, hDPSC induction may appear after carious or traumatic injuries and the subsequent application of a restorative material. All these events lead to an inflammatory reaction, which is believed to be the initial step of tissue regeneration (Kloner et al. 1978; Mitsiadis et al. 2008). This pinpoints the fact that understanding what happens in an inflammatory environment is essential in understanding the mechanism of DPSC OD in the dentin-pulp regeneration processes. Accordingly, we decided to evaluate the effect of 5 proinflammatory components (LPS, LTA, IL6, TNF α , and C5a), known to be involved in the dental-pulp inflammation, in the expression of C5L2 by hDPSCs (Loesche 1986; Martin et al. 2002; Chmielewsky et al. 2014; Farges et al. 2015). Interestingly, our data revealed that TNF α affects the expression of C5L2 by hDPSCs, by specifically increasing its expression during the OD. Further analysis showed that the odontogenesis-regulated expression of C5L2 quickly increased (only 6-h differentiation) and is sustained with increasing concentration of TNF α treatment. Comparatively, none of these proinflammatory components affects the expression of C5aR by hDPSCs. These data highlight the importance to consider the “inflammation” parameter in the study of the biology of DPSCs during the differentiation process and supports the potential critical implication of C5L2 in the OD. In addition, although the expression of C5L2 appears to be increased in hBMMSCs undergoing osteogenic differentiation, TNF α stimulations do not affect the level of C5L2 during the differentiation process, regardless of the concentration used. These results are consistent with the possibility that C5L2 is involved in stem cell differentiation and clearly indicated that the C5L2 expression in response to TNF α depends on the organ/tissue considered, thereby pinpointing the importance of both the “inflammation” parameter and the targeted cell type in dentin-pulp regeneration.

DMP1 is a noncollagenous protein secreted by the odontoblasts and is directly implicated in the nucleation and the control of the growth of the mineral phase (George et al. 1993). Because the *DMP1* gene is highly expressed in odontoblasts

during tooth development and dentin formation, it is considered a key marker of the hDPSC OD and mineralization (Martinez et al. 2009). Interestingly, our immunohistochemistry experiment revealed that C5L2 staining is more intense in the dental pulp of mice genetically modified to overexpress DMP1 as compared with wild-type mice, indicating a possible link between DMP1 expression and C5L2. A recent study conducted in human dental pulp fibroblasts demonstrated that the silencing of C5L2 induced an upregulation of BDNF secretion (brain-derived neurotrophic factor) by LTA-stimulated pulp fibroblasts (Chmielewsky et al. 2017); further, it was demonstrated that C5L2 silencing enhanced nerve outgrowth in human pulp fibroblasts when stimulated by LPS (Chmielewsky et al. 2018). Accordingly, we found that the silencing of C5L2 expression in hDPSCs significantly increased the DMP1 expression in hDPSCs undergoing OD. Although the upregulation of C5L2 in DMP1-overexpressed mice and DMP1 in C5L2-silenced DPSCs seems to be opposite, further analysis revealed that the stimulation of DPSCs with recombinant DMP1 significantly increased the expression of C5L2. Taken together these data could indicate a loop of regulation between C5L2 and DMP1, where DMP1 could be a positive regulator of C5L2 and C5L2, a negative regulator of DMP1. The analysis of DMP1-overexpressed mice at different stages and the development of an in vivo model of mice genetically modified for C5L2 expression could constitute an interesting way to evaluate this regulation loop. It was previously reported that TNF α increases the early expression of DMP1 in human dental pulp cells (Paula-Silva et al. 2009). Accordingly, our data revealed that while TNF α stimulation significantly increases the DMP1 expression in undifferentiated hDPSCs, it abolishes the DMP1 upregulation in hDPSCs undergoing OD. Interestingly, we found out that in the presence of TNF α , demonstrating elevated C5L2 expression, the silencing of C5L2 potentiated the increase of DMP1 expression by hDPSCs undergoing OD. The 2 opposite effects of TNF α on the expression of C5L2 and DMP1 are consistent with our results demonstrating that C5L2 constitutes a negative regulator for DMP1. Finally, we showed that the stimulation of hDPSCs with recombinant human C5a has no effect on the DMP1 expression. This result is in accordance with our data showing an odontogenesis-regulated expression of C5L2 versus C5aR. Indeed, both these data suggest a C5a/C5aR-independent function of C5L2 in hDPSC differentiation. Taken together, these results clearly demonstrated that C5L2 constitutes a negative regulator of the DMP1 expression during the OD of hDPSCs.

In summary, our data present a novel insight into the biological function of C5L2 during dentinogenesis in the presence of proinflammatory cytokine TNF α . This work is of importance as C5L2 functions as a negative regulator of DMP1 expression by hDPSCs during OD differentiation and inflammation processes, suggesting a direct correlation between OD and C5L2 expression in hDPSCs. This work, which is the first to demonstrate the involvement of C5L2 in the biological function of stem cells, may provide a useful future therapeutic tool in targeting C5L2 to improve the dentin-pulp regeneration process.

Author Contributions

F. Chmilewsky, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; R. Liang, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; M. Kanazawa, contributed to data acquisition and analysis, critically revised the manuscript; L.F. Cooper, contributed to conception, design, data interpretation, critically revised the manuscript; I. About, A. George, contributed to conception, design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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